

AD _____

Award Number: DAMD17-00-1-0556

TITLE: New Anti-Metastatic and Anti-Angiogenic Compound for
Ovarian Cancer

PRINCIPAL INVESTIGATOR: Erkki I. Ruoslahti, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

REPORT DATE: September 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20021001 050

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

V Where copyrighted material is quoted, permission has been obtained to use such material.

V Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

V Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

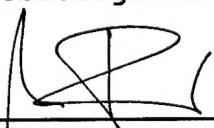
V In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A V For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NA V In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

NA V In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

NA V In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature Date
Erkki Ruoslahti, M.D., Ph.D.

7.31.01

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 2001	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 00 - 31 Aug 01)	
4. TITLE AND SUBTITLE New Anti-Metastatic and Angi-Angiogenic Compound for Ovarian Cancer			5. FUNDING NUMBERS DAMD17-00-1-0556	
6. AUTHOR(S) Erkki I. Ruoslahti, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Burnham Institute La Jolla, California 92037 E-Mail: ruoslahti@burnham.org			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Report contains color				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) We have developed a novel substance, a polymeric form of fibronectin that we have named sFN, which has profound anti-tumor activities. Importantly, there seems to be no toxicity associated with systemic sFN treatment, even when given over several months. At least two characteristics of sFN contribute to its anti-tumor effects: it is both anti-angiogenic and anti-metastatic. sFN is a complex of fibronectin itself and a fragment from fibronectin, anastellin, which binds tightly to fibronectin and causes polymerization of fibronectin. We have now found that anastellin alone reproduces the anti-angiogenic effects of sFN and that plasma fibronectin is needed for anastellin to work in vivo. This is an important advance in understanding how anastellin (and other anti-angiogenic proteins) function. sFN is particularly effective in suppressing the growth and spreading of experimental ovarian cancer in mice. Anastellin, which would be easier to produce and administer may be a better compound to attempt to advance into clinical trials.				
14. SUBJECT TERMS Cancer Biology/Therapy: Angiogenesis, tumor invasion, endothelial cell biology, metastasis				15. NUMBER OF PAGES 13
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		20. LIMITATION OF ABSTRACT Unlimited

4. Table of Contents

Cover	1
SF298	2
Foreword	3
Table of Contents	4
Introduction	5
Body	5
Key Research Accomplishments	7
Reportable Outcomes	7
Conclusions	8

Appendix: Yi and Ruoslahti, PNAS 98:620-624, 2001

5. INTRODUCTION

We have developed a novel substance, a polymeric form of fibronectin that we have named sFN, which has profound anti-tumor activities. Importantly, there seems to be no toxicity associated with systemic sFN treatment, even when given over several months. At least two characteristics of sFN contribute to its anti-tumor effects: it is both anti-angiogenic and anti-metastatic. sFN is a complex of a fragment from fibronectin (which binds tightly to fibronectin) and fibronectin itself. The sFN-inducing fragment alone reproduces the anti-tumor effect of sFN and have named this fragment 'anastellin' (Yi and Ruoslahti, 2001). Anastellin is thought to form a functional sFN complex with fibronectin present in blood. There are striking similarities between anastellin and the other known anti-angiogenic compounds: heparin binding and the ability to make complexes with adhesion proteins that interact with the $\alpha v \beta 3$ integrin. This integrin is essential to the survival of angiogenic endothelial cells. We proposed in the original application to experimentally test of the hypothesis that sFN and the known protein inhibitors of angiogenesis share a mechanism of action, and that this mechanism is to bind to the $\alpha v \beta 3$ integrin and somehow disrupt the endothelial cell supporting functions of this integrin. We have found sFN is particularly effective in suppressing the growth and spreading of experimental ovarian cancer in mice, and clinical trials are planned in this cancer. Thus, these studies are relevant to cancer in general and ovarian cancer in particular.

6. BODY

The approved tasks for this project include:

Task 1. To develop conditions for preparing (1) antithrombin-vitronectin complexes, (2) fibrinogen complexes made in a manner analogous to the preparation of sFN, and (3) sFN at different degrees of polymerization.

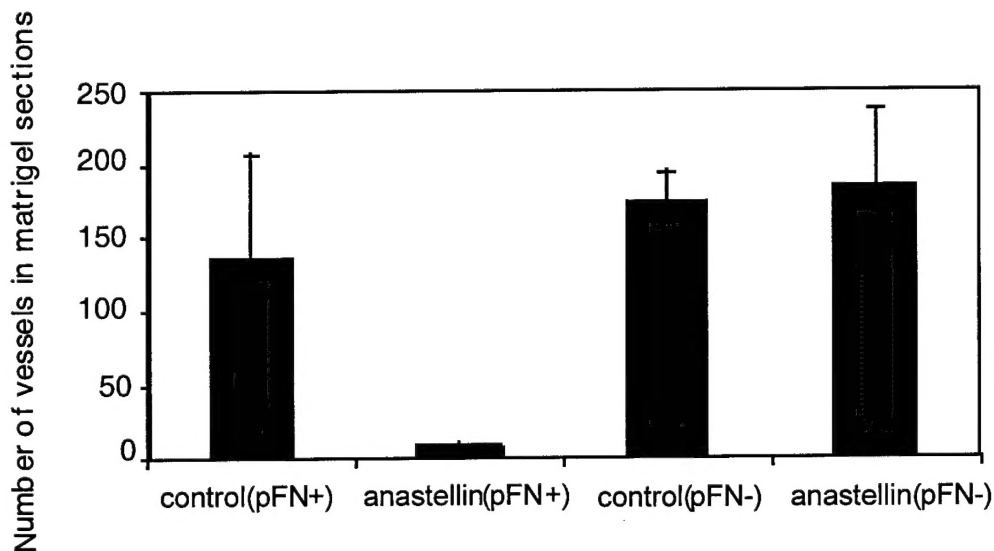
Task 2. To study the effects of the above reagents on endothelial cell proliferation, apoptosis and migration, and angiogenesis on chorioallantoic membrane (CAM).

Task 3. To test the most promising materials from Task 2 for inhibition of tumor growth in a mouse ovarian cancer model.

During this first year of grant support we have made substantial progress with Tasks 1(2), 1(3) and 2.

Task 1(2 and 3). We have shown that anastellin can cause polymerization of fibrinogen. Analysis of the polymerization conditions showed that more anastellin is needed to obtain the same degree of polymerization as with fibronectin, and that the fibrinogen concentration should be higher than that of fibronectin. These results, which complete Task 1(2 and 3), have been published in the appended article (Yi and Ruoslahti, 2001), which appeared in PNAS (Figure 1 in that article, attached).

We have also generated a potentially important preliminary result under Task 1(2) indicating that fibronectin is needed for anastellin to be active in vivo. After the original application was submitted, a two mouse cell lines that are conditionally deficient in plasma fibronectin were published (Sakai et al., 2001). This system gave us the opportunity to study a situation where there is no fibronectin polymerization. We obtained the mice and have so far used one of the strains to show that anastellin has no anti-angiogenic effect in vivo in these mice (Figure, left two columns). We first showed that anastellin inhibits non-tumor angiogenesis in a matrigel angiogenesis assay (Fulgham et al., 1999; Ngo et al., 2000). In this assay, a bolus of extracellular matrix gel (matrigel) is injected subcutaneously. Matrigel is an injectable liquid at 4°, but solidifies into a pellet at body temperature. If spiked with an angiogenic factor, such as bFGF, it attracts angiogenic neovasculature that grows into the gel within a week or so. Systemic administration of anastellin caused nearly complete inhibition of angiogenesis in this system, whether the angiogenesis stimulant was bFGF (see Figure) or VEGF (not shown). Importantly, the inhibitory activity of anastellin was not seen in mice that lack plasma fibronectin (Figure, two columns to the right).



Anastellin is inactive in mice that lack plasma fibronectin. Matrigel pellets were implanted in mice engineered to lose plasma fibronectin after birth (floxed fibronectin gene with Cre under the albumin promoter; Sakai et al., 2001) and in their normal littermates. Matrigel pellets impregnated with bFGF were implanted subcutaneously into mice and the mice were treated with 1 mg of anastellin as daily intraperitoneal injections starting on the day after the implantation. The pellets were removed on day 8 after the implantation and blood vessel density in them was quantified under the microscope in sections stained with anti-CD31 for the general blood vessel marker CD31.

The observation that anastellin is ineffective in mice that are deficient in plasma fibronectin supports the hypothesis presented in the original application that fibronectin binding as a critical element in anastellin activity. Because these mice have plasma fibrinogen, this result also shows that fibrinogen binding by anastellin is not sufficient for substantial *in vivo* activity of anastellin. The *in vivo* activity we observed with *ex vivo* prepared anastellin-fibrinogen complexes (Yi and Ruoslahti, 2001) is probably mostly due to the presence of excess anastellin in the complexes. Transfer of anastellin to fibronectin, which has a higher affinity for it, may also be a factor *in vivo*. These new results validate our plan to study matrix protein complexes. As indicated in the application, we will next analyze antithrombin-vitronectin complexes in a similar manner.

Task 2. As shown above, we have developed the necessary methodology to assess the anti-angiogenic activity of our candidate proteins *in vivo*. We have compared the CAM assays we originally proposed to use and the matrigel assays (Pasqualini et al., 2000; Yi and Ruoslahti, 2001). Our experience is that the matrigel assay gives steadier results. Moreover, because the matrigel assays can be performed in adult mice, rather than in chicken eggs, matrigel assays are likely to better reflect the human pathophysiology than CAM assays. The results obtained in the matrigel system are going to be particularly useful in designing the experiments for Task 3, which we will start working on in the coming year.

7. KEY RESEARCH ACCOMPLISHMENTS

We have demonstrated:

- that anastellin (III1-C) polymerizes fibrinogen the same way it was previously known to polymerize fibronectin (to form sFN).
- that anastellin alone (without exogenously added fibronectin) is effective as an anti-angiogenic factor *in vivo*.
- that anastellin is ineffective in mice that lack plasma fibronectin, showing that complexing with fibronectin is important (and complexing with fibrinogen is not) for the *in vivo* activity.
- that a matrigel angiogenesis assay is suitable for the analysis of the *in vivo* anti-angiogenic properties of anastellin (and sFN).

8. REPORTABLE OUTCOMES

Publication:

Yi and Ruoslahti, Proc. Natl. Acad. USA, 2001 (enclosed) reports some of the results relating to Task 1(2 and 3). Most of the work accomplished during the report year is still unpublished.

Patent application:

Ruoslahti, E. and Yi, M. "Methods of inhibiting tumor growth and angiogenesis with anastellin". U.S. Patent application filed December 4, 2000.

9. CONCLUSIONS

We have made significant progress toward understanding the mechanism of action of sFN and its key component, the III-C fibronectin fragment now known as anastellin. Particularly important was our (still preliminary) result to the effect that anastellin is ineffective in mice that lack plasma fibronectin. This result suggests that fibronectin polymerization is important for anastellin activity, and that the ability of anastellin to polymerize fibrinogen is not important. The matrigel assay we have adopted to our studies has already proven to be a productive tool for us.

References

- Fulgham, D.L., Widhalm, S.R., Martin, S. and Coffin, J.D. FGF-2 dependent angiogenesis is a latent phenotype in basic fibroblast growth factor transgenic mice. *Endothelium* 6:185-195 (1999).
- Ngo, C.V., Gee, M., Akhtar, N., Yu, D., Volpert, O., Auerbach, R. and Thomas-Tikhonenko, A. An in Vivo Function for the Transforming Myc Protein: Elicitation of the Angiogenic Phenotype. *Cell Growth Differentiation* 11:201-210 (2000).
- Yi, M and Ruoslahti, E. A fibronectin fragment inhibits tumor growth, angiogenesis, and metastasis. *Proc. Natl. Acad. Sci. USA* 98:620-624 (2001). **APPENDED**
- Sakai, T., Johnson, K.J., Murozono, M., Sakai, K., Magnuson, M.A., Wieloch, T., Cronberg, T., Isshiki, A., Erickson, H.P. and Fassler, R. Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. *Nature Med.* 7: 324-30 (2001).
- Pasqualini, R., Koivunen, E., Kain, R., Lahdenranta, J., Sakamoto, M., Stryhn, A., Ashmun, R.A., Shapiro, L.H., Arap, W., and Ruoslahti, E. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Res.* 60: 722-727 (2000).

A fibronectin fragment inhibits tumor growth, angiogenesis, and metastasis

Ming Yi and Erkki Ruoslahti*

Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037; and Molecular Pathology Graduate Program, University of California, San Diego, CA 92093

Contributed by Erkki Ruoslahti, October 16, 2000

We have shown previously that a polymeric form of fibronectin is strongly antimetastatic when administered systemically to tumor-bearing mice. The polymeric fibronectin, sFN, is formed *in vitro* by treating soluble fibronectin with a 76-aa peptide, III1-C, which is derived from the first type III repeat in fibronectin. Here we show that the III1-C peptide and sFN also reduce tumor growth in mice, and that this effect correlates with a low density of blood vessels in the tumors of the treated mice. III1-C also polymerized fibrinogen, and the fibrinogen polymer, sFBG, had antitumor and antiangiogenic effects similar to those of sFN. Mice that had been injected s.c. with three different types of human tumor cells and treated with biweekly i.p. injections of III1-C, sFN, or sFBG over a 5-week period had tumors that were 50–90% smaller than those of control mice. Blood vessel density in the tumors of the treated mice was reduced by 60–80% at the end of the experiment. Xenograft tumors from a human breast carcinoma line (MDA-MB-435) were particularly susceptible to these treatments. Metastasis into the lungs from the primary s.c. tumors also was inhibited in the mice treated with III1-C and the two polymers. The III1-C peptide is an antiangiogenic and antimetastatic agent. Because of its ability to suppress tumor growth, angiogenesis, and metastasis, we have named the III1-C peptide anastellin [from *anastello* (Greek), inhibit, force a retreat].

Fibronectin is a prototypic extracellular matrix (ECM) protein that is deposited by various types of cells into an adhesive fibrillar meshwork of protein (1). Fibronectin, and ECM in general, control many cellular functions, including growth, migration, differentiation, and survival. The signals that control these behaviors are transmitted from the ECM to the cell by integrins, a family of transmembrane receptors (2, 3). Malignant cells often bypass the ECM–integrin signaling system; they are not bound by the spatial constraints imposed by the ECM on normal cells, and they no longer require ECM contact for survival (4).

In past studies, we have looked for ways to restore the matrix control in malignant cells and developed a polymeric form of fibronectin, sFN (5). sFN is generated by treating soluble fibronectin with a 76-aa fibronectin fragment, the III1-C peptide, anastellin, which comes from the first type III repeat of the fibronectin polypeptide (5). The first type III repeat contains a site that is important in fibronectin self-assembly into fibrils (6–8). The isolated III1 repeat and its fragments may interfere with the intramolecular binding interactions that keep fibronectin in its soluble configuration (7), and disruption of those interactions may induce the molecule to undergo assembly into fibrils (5). Alternatively, the III1 fragments could change the conformation of the fibronectin molecule in such a way that cryptic fibronectin–fibronectin binding sites that are capable of driving fibril assembly are exposed (9). The III1 fragments used to induce sFN formation remain part of the sFN polymer. The polymer is 10-fold more strongly adhesive to cells than fibronectin coated onto plastic without polymerization (5). We also have shown that sFN has profound antimetastatic effects when administered systemically to mice bearing various types of tumors (10). The sFN-inducing fragment, anastellin, was tested in

parallel with sFN, but it had only a marginal effect on metastasis. The antimetastatic effects were obtained without any significant reduction in the growth rate of the primary tumors.

Because these early *in vivo* studies were focused on metastasis, they used animals carrying large primary tumors. The use of sFN and anastellin to treat smaller tumors at higher doses has now revealed additional antitumor activities in these compounds. We show here that anastellin and sFN curtail both the growth and metastasis of various types of xenograft tumors in mice and that the antitumor activity of these compounds is related to their ability to inhibit tumor angiogenesis.

Materials and Methods

Proteins. Anastellin (the III1-C fibronectin fragment) and III11-C (control fragment from the 11th type III repeat) were prepared as recombinant His-tagged proteins in bacteria and purified as described (5, 10). Human plasma fibronectin was from Chemicon, and human fibrinogen was from Sigma. We converted fibronectin to sFN by mixing 100 μ g of fibronectin in 100 μ l PBS with 300 μ g of anastellin in 100 μ l PBS (10). III1-C also binds to fibrinogen (7), and substitution of fibrinogen (from human plasma, fraction I; Sigma) for fibronectin yielded polymerized fibrinogen (“superfibrinogen,” sFBG). Protein polymerization was monitored by measuring the optical density at 620 nm. The sFN and sFBG preparations were freshly made for each experiment. The protein solutions were sterilized by filtering through 0.2- μ m membrane before polymerization.

Tumor Cell Culture and Harvesting. The C8161 melanoma, KRIB osteosarcoma, and MDA-MB-435 breast carcinoma human tumor cell lines were used to establish human xenograft tumors in nude mice as described (10, 11). Before use, the cells were grown in the continuous culture for no more than three consecutive passages. Actively growing cells were detached from culture plates with PBS/2.5 mM EDTA or trypsin-EDTA (0.25% trypsin/1 mM Na-EDTA; GIBCO/BRL). The detached cells were resuspended in DMEM, counted, and examined for viability by trypan blue exclusion. The cells were injected into mice as described in the next section. A portion of the cells that were used in the injections was seeded back into a culture plate to determine plating efficiency. The viability was higher than 99%, and the plating efficiency was greater than 95%.

Tumor Inoculation. Two-month-old immunodeficient BALB/c *nu/nu* female mice (Harlan–Sprague–Dawley) were used for the experiments. To obtain s.c. tumors, we injected 10^6 tumor cells in 200 μ l of DMEM into the right posterior flank of the mice;

Abbreviations: ECM, extracellular matrix; sFN, polymeric fibronectin; sFBG, fibrinogen polymer (“superfibrinogen”).

*To whom reprint requests should be addressed. E-mail: ruoslahti@burnham.org.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

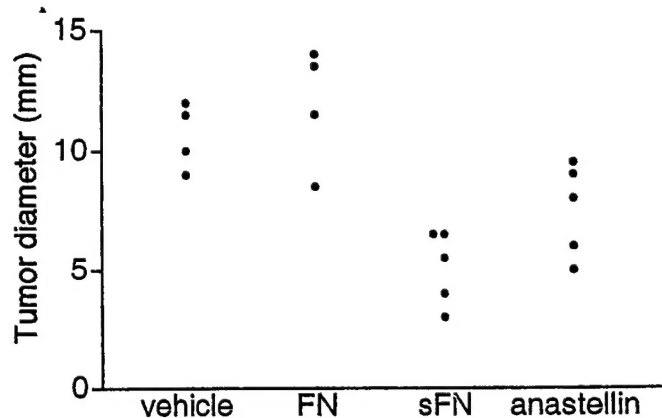


Fig. 1. Effect of systemic treatment with anastellin on the growth of human xenograft tumors in mice. s.c. tumors were grown in nude mice from C8161 melanoma cells. Treatment with biweekly i.p. injections of the indicated substances was started 3 weeks after the tumor implantation and continued for 3 weeks. The injections consisted of vehicle, 100 μ g of fibronectin, 100 μ g of fibronectin mixed with 300 μ g of anastellin (sFN), or 300 μ g of anastellin alone. The tumors of the mice treated with sFN or anastellin grew significantly less than the tumors of the mice treated with vehicle alone ($P < 0.001$ and $P < 0.05$, respectively).

the mice were randomized and divided into experimental groups, with five or six mice per group. At 3 weeks after the tumor cell implantation, when nearly all of the mice had developed palpable tumors, the mice were treated with i.p. injections (200 μ l) of various proteins in PBS or with PBS alone. The treatments were administered twice a week until the experiment was terminated.

To monitor tumor growth during the treatment, we estimated tumor size by measuring biweekly the longest and shortest diameters of the tumor and by averaging the two measurements. At the end of the experiment (usually 8 weeks after tumor cell implantation and 5 weeks after the start of treatments), the mice were anesthetized and perfused through the heart with PBS, and the tumors and the lungs were excised and weighed. The lungs also were examined for the number of metastatic foci. The tissues were fixed in 4% paraformaldehyde for 24 h and stored in 70% ethanol. Paraffin embedding, sectioning, and immunostaining for blood vessels with anti-CD31 were carried out in the Burnham Institute Histology Facility. The rat anti-mouse CD31 antibody was from PharMingen.

Results

Systemic Treatment with sFN or the sFN-Inducing Anastellin Peptide Inhibits Tumor Growth. In previous work, we found that systemic treatment with sFN, a polymer of fibronectin that is prepared by mixing fibronectin and anastellin, had a strong antimetastatic effect when used to treat mice bearing large (0.5 g in weight) tumors. This effect was achieved even though sFN had little or no effect on the growth of the primary tumors (10). In the present study, we started the treatments at an earlier stage of tumor development and found that sFN significantly inhibited the growth of C8161 human melanoma xenograft tumors (Fig. 1). Surprisingly, anastellin given alone at the dose used to prepare sFN had a similar, albeit perhaps somewhat weaker, antitumor activity as sFN.

Anastellin-Induced Fibrinogen Polymer. Our finding that anastellin could inhibit tumor growth without being mixed *ex vivo* with fibronectin raised the question of whether anastellin might form an active complex with fibrinogen *in vivo*. A fibronectin fragment that overlaps with anastellin binds to both fibrinogen and

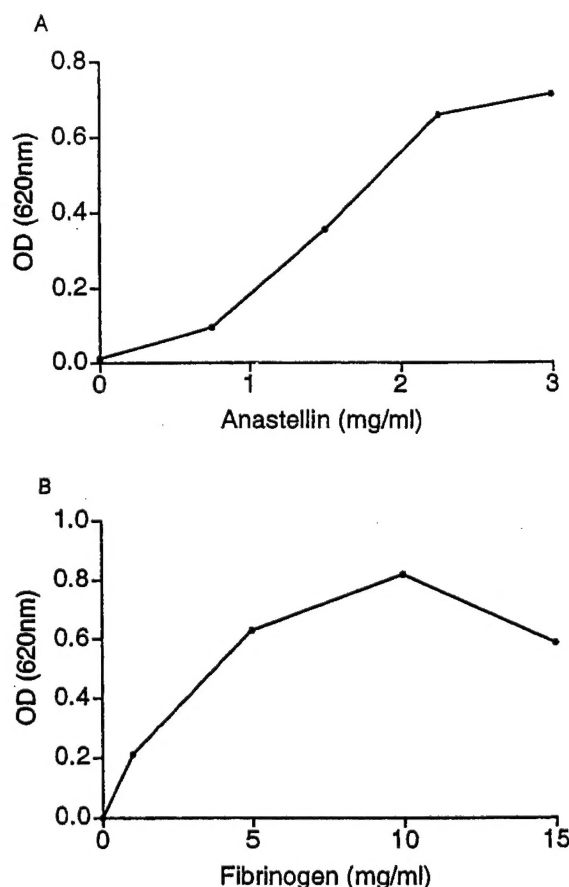


Fig. 2. Polymerization of fibrinogen by anastellin. Anastellin and fibrinogen at various concentrations were mixed 1:1 (vol/vol), and protein polymerization was followed by monitoring optical density at 620 nm (turbidity). (A) An increase in turbidity is seen when a constant amount of fibrinogen (5 mg/ml) is mixed with increasing amounts of anastellin. (B) A constant amount of anastellin (3 mg/ml) was mixed with an increasing amount of fibrinogen. Mixing fibrinogen with a control fragment from the 11th type III repeat of fibronectin (III-11C; 3 mg/ml) yielded essentially baseline turbidity (data not shown).

fibronectin (7), and fibrinogen is more abundant in plasma than is fibronectin. Indeed, we found that adding anastellin to a fibrinogen solution *in vitro* caused turbidity, producing a polymeric compound, sFBG (Fig. 2). However, a higher concentration of fibrinogen than fibronectin was needed to produce the same degree of polymerization with anastellin. In subsequent tumor treatment experiments, we compared sFN and sFBG at the same approximate levels of polymerization. The solubility of fibronectin limited the amount of sFN that could be administered to a mouse, but anastellin alone does not have this limitation. Thus, we also tested anastellin at a higher dose.

Comparison of the Antitumor Effects of Anastellin, sFN, and sFBG. Comparing the antitumor activities of the various anastellin compounds, we found that sFBG inhibited tumor growth approximately as effectively as anastellin alone or sFN. Similar results were obtained with three different tumors, C8161 (not shown), KRIB human osteosarcoma (Fig. 3), and MDA-MB-435 human breast carcinoma (Fig. 4). In some experiments, a peptide analogous to anastellin but derived from the 11th type III repeat of fibronectin (III11-C) was used as an additional control. This peptide does not bind to fibronectin (5) and had no effect on the growth of the breast carcinoma tumors (Fig. 4). In other experiments, we also included as controls nonpolymerized fibronectin and fibrinogen at the same

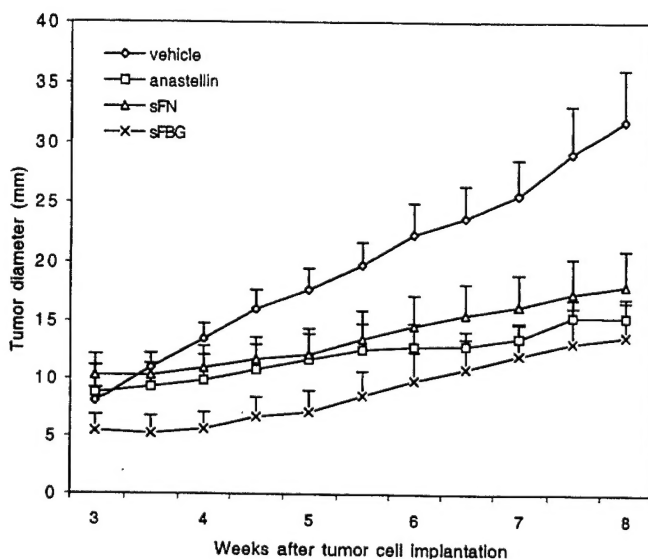


Fig. 3. Inhibition of KRIB osteosarcoma growth by anastellin, sFN, and sFBG. s.c. tumors were grown in nude mice from KRIB osteosarcoma cells. Treatment by i.p. injections of the indicated substances (six mice per treatment group) were started 3 weeks after tumor implantation, repeated twice a week, and continued for 5 weeks. Each injection consisted of 100 μ g of fibrinogen mixed with 300 μ g of anastellin (sFN), 500 μ g of fibrinogen mixed with 300 μ g of anastellin (sFBG), or 600 μ g of anastellin. Tumor sizes (mean and SEM) in each treatment group (six mice per group) at the indicated time point are shown. The tumors of the mice treated with anastellin, sFN, or sFBG were significantly smaller at the end of the experiment than those of the mice treated with vehicle alone (anastellin, $P < 0.01$; sFN, $P < 0.05$; sFBG, $P < 0.01$).

dose as that used in the polymers. They had no effect on tumor growth (Fig. 1 and results not shown).

Anastellin, sFN, and sFBG Inhibit Tumor Angiogenesis. We hypothesized that the decreased tumor growth obtained with anastellin, sFN, and sFBG could be caused by inhibition of tumor angio-

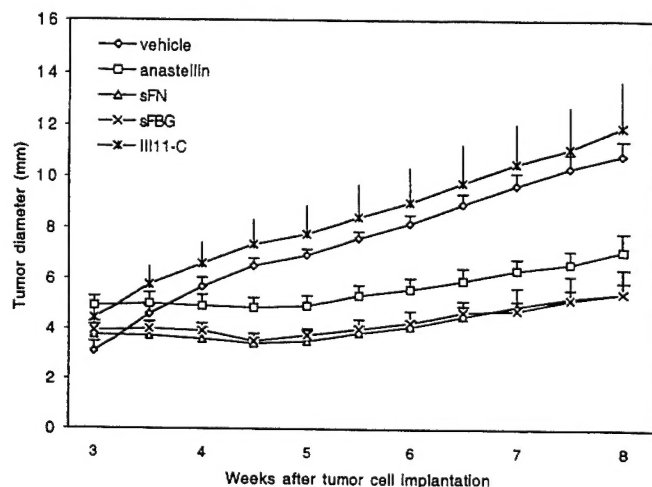


Fig. 4. Inhibition of MDA-MD-435 breast carcinoma growth by anastellin, sFN, and sFBG. Nude mice bearing s.c. tumors from MDA-MB-435 breast carcinoma cells were used in a treatment experiment similar to the one shown in Fig. 3. The doses per injection were as follows: anastellin, 600 μ g; sFN, 100 μ g of fibrinogen mixed with 300 μ g of anastellin; sFBG, 500 μ g of fibrinogen mixed with 300 μ g of anastellin; III-11C, 600 μ g. Six mice were used per group. The tumors were significantly smaller at the end of the experiment in the mice treated with anastellin ($P < 0.01$), sFN ($P < 0.001$), or sFBG ($P < 0.001$) than in the mice treated with vehicle alone or with III-11C.

Table 1. Number of mice with lung metastases among mice bearing C8161 or KRIB tumors and treated with various anastellin compounds

Tumor	Vehicle	Anastellin	sFN	sFBG
C8161	17/17	4/11**	1/11***	—
KRIB	9/12	3/12*	4/12*	5/12†

Mice with KRIB and C8161 tumors from the experiments shown in Figs. 3 and 4 and other similar experiments were examined for macroscopic lung metastases at the end of the experiments (or at the time of death, if earlier). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ relative to the vehicle group.

†The sFBG group was not significantly different from the control group ($P = 0.11$), but the number of metastatic foci (not shown) was significantly smaller than in the controls ($P < 0.01$).

genesis. We therefore analyzed the density of blood vessels by staining sections of tumors collected at the end of the experiments shown in Figs. 1, 3, and 4. Staining for the endothelial marker CD31 showed greatly reduced blood vessel density in the tumors of the mice treated with anastellin, sFN, and sFBG relative to the vehicle-treated controls. The results were similar for all of the three tumor types; Fig. 5 shows examples of the staining results with KRIB tumors, and quantitative results are shown for the MDA-MB-435 tumors in Fig. 6. In contrast to the tumor results, the density of CD31 positive vessels in the lungs of the mice that received the various treatments showed no discernible differences (not shown). These results suggest that the inhibition of tumor growth by anastellin, sFN, and sFBG is caused at least in part by suppression of tumor angiogenesis.

Anastellin, sFN, and sFBG Inhibit Metastasis. We also examined the lungs of the tumor-bearing mice for evidence of metastasis. All of the vehicle-treated mice with C8161 tumors and most of the mice with KRIB tumors developed macroscopic lung metastases; the MDA-MB-435 tumors did not metastasize within the time frame of our experiments. In agreement with earlier results, only a few mice with C8161 or KRIB tumors developed metastatic foci in the lungs when treated with sFN (10) or anastellin (Table 1). sFBG was studied less extensively but seemed to have an effect on metastasis similar to that of sFN and anastellin.

Discussion

We describe here a antiangiogenic protein, anastellin. Anastellin is a fibrinogen fragment that binds to and polymerizes fibrinogen and fibrinogen. Our results show that systemic treatment of mice with intraperitoneally injected anastellin or its fibrinogen or fibrinogen polymers suppresses the growth of s.c. tumors and that this effect of anastellin is likely to be caused by its antiangiogenic activity. Anastellin also inhibits metastasis.

We previously have shown that sFN, a fibrinogen polymer induced by anastellin, has a potent antimetastatic activity in experimental and spontaneous metastasis models (10). In that study, systemic treatment of the tumor-bearing mice with sFN or anastellin had no significant effect on tumor growth. In contrast, we found in the present study that tumor growth was inhibited by these compounds, as well as by sFBG, a fibrinogen polymer generated by treating soluble fibrinogen with anastellin. The likely explanation for the difference between the previous result and the antitumor effect we observed here is in the timing of the treatment. In the metastasis study, the tumors were large at the time when the treatment was started, whereas smaller tumors were treated in the present study. The large tumors might have been beyond the point where antiangiogenic treatment would still be effective. Furthermore, the dose might not have been optimal. Although a complete dose-response study remains to

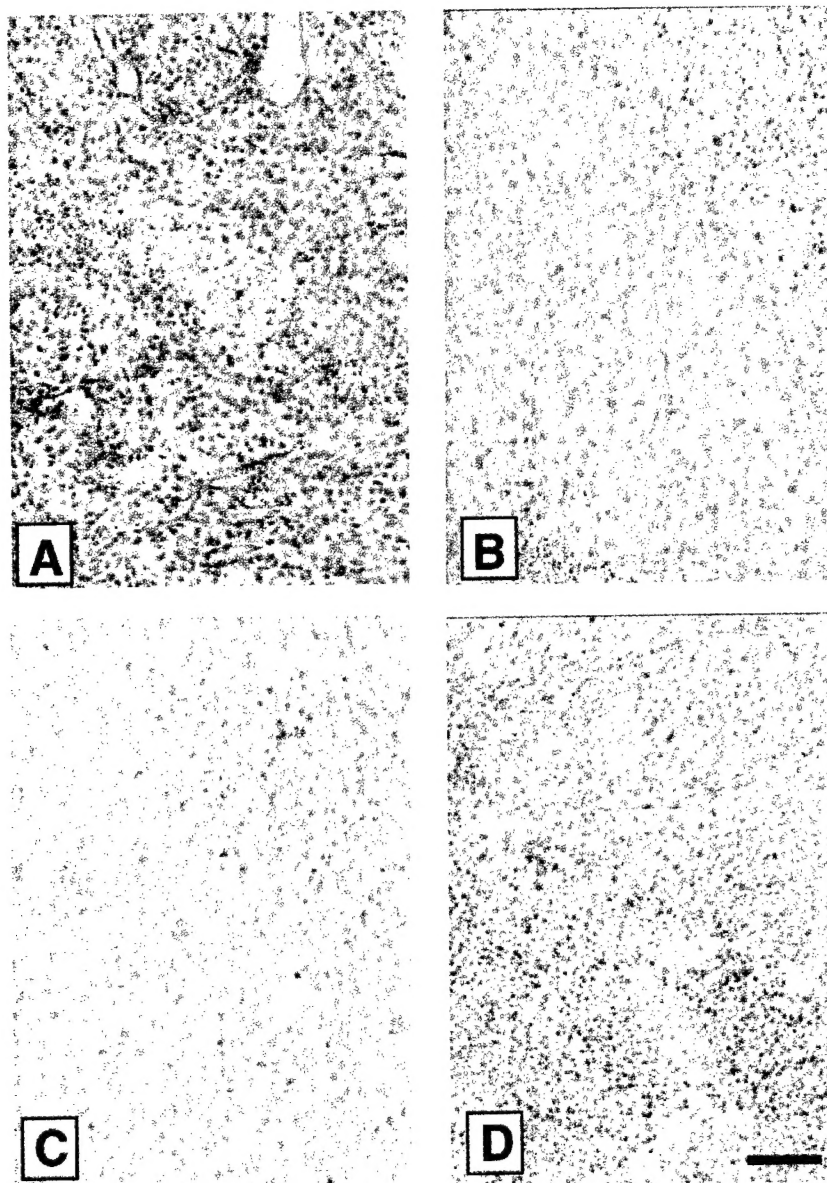


Fig. 5. Immunohistochemical staining for blood vessels in sections of KRIB tumors. KRIB osteosarcoma tumors from a treatment experiment similar to the one shown in Fig. 3 were removed at the end of the experiment and sectioned, and the sections were stained with anti-CD31 antibodies to visualize tumor blood vessels. Representative microscopic fields from the tumors show a higher density of blood vessels in the vehicle alone group (A) than in the anastellin (B), sFN (C), and sFBG (D) groups (Magnification, $\times 400$; bar = 50 μm .)

be done, the present results suggest that doubling the dose of anastellin made it more potent.

Our results indicate that the inhibitory effect of anastellin and the fibronectin and fibrinogen polymers is mediated by an antiangiogenic activity of these compounds. This effect seems to be different from the antimetastatic activity we observed earlier (10), where only sFN was clearly active. Here, anastellin was approximately as effective as sFN in suppressing tumor growth and tumor angiogenesis.

The antiangiogenic effects of anastellin, sFN, and sFBG are likely to have been primarily responsible for the inhibition of tumor growth that we have demonstrated here. The blood vessel density in the tumors of the treated mice was only about 20% of that in control tumors. As vascularization is a prerequisite of tumor growth (12), the low number of blood vessels must have been a major impediment to tumor growth in the treated mice.

Inhibition of angiogenesis with its suppressive effect on tumor growth possibly underlies the antimetastatic effect of

anastellin and the polymers we observed in this study. First, we found that the number of metastases correlated with the size of the primary tumor and the number of blood vessels in it. The reduced vasculature in the tumor could make it more difficult for tumor cells to enter the circulation. Another possible contributing factor is the reduced ability of tumor cells that have gained access to the circulation to establish metastatic colonies. Earlier studies have shown that sFN inhibits lung colonization by tumor cells injected into the circulation (10). As anastellin exhibited this activity, or did so weakly, this inhibitory activity could explain why sFN might be somewhat more active in reducing metastasis than is anastellin, at least with the C8161 tumors. Anastellin also did not inhibit spontaneous metastasis in the previous study, suggesting that the antimetastatic activities of sFN observed in the previous study depended on a mechanism other than antiangiogenic activity. Preliminary data suggest that that effect is related to accelerated removal of tumor cells from the circulation (unpub-

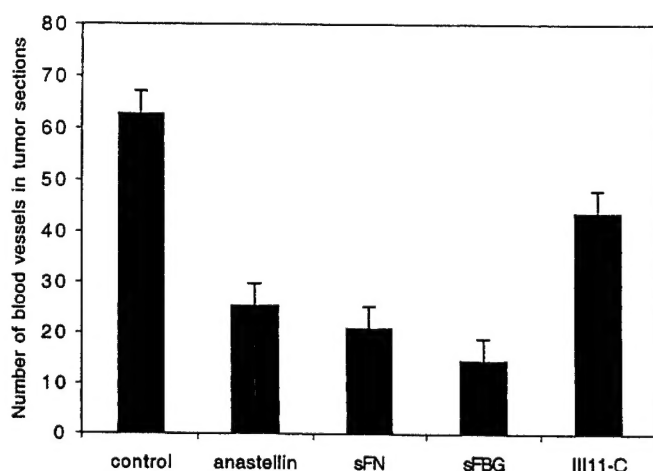


Fig. 6. Quantification of decreased blood vessel density in MDA-MB-435 tumors from mice treated with anastellin, sFN, or sFBG. Blood vessels in tumor sections stained with anti-CD31 were counted from five microscopic fields for all tumors in the experiment shown in Fig. 4. The mean and SEM for the number of blood vessels in the tumors from the six mice in each treatment group are shown. The blood vessel density was significantly reduced in each of the anastellin ($P < 0.02$), sFN ($P < 0.02$), and sFBG ($P < 0.01$) groups relative to the vehicle alone group and the other control group that received III-11C.

lished results). Thus, it is possible that the antitumor effects of anastellin and sFN are not completely overlapping.

Anastellin is a representative of a growing class of antiangiogenic substances that are derived from ECM and blood proteins

by proteolysis or other modifications. These substances include angiostatin (13), endostatin (14), heparin-binding fragments of fibronectin (15, 16), and a modified form of antithrombin (17). As is the case with these substances, the mechanism of the antiangiogenic activity of anastellin is unknown. However, we do know that each of these substances binds to one or more adhesion proteins. Angiostatin and its parent protein plasminogen can bind vitronectin (18), endostatin has been shown to bind to fibulins and nidogen-2 (19), and the antiangiogenic form of antithrombin is similar to the modified antithrombin that binds to vitronectin (20). Anastellin not only binds to fibronectin and fibrinogen in serum (7), it polymerizes these proteins *in vitro* (ref. 5 and the present study) and likely *in vivo*. Fibronectin and fibrinogen, and each of the other ligands for the various antiangiogenic substances we have listed, are adhesion proteins containing the arginine-glycine-aspartic acid cell adhesion sequence (21). Moreover, they all bind to the $\alpha v \beta 3$ integrin, which is expressed at high levels in angiogenic endothelial cells and which plays an important role in angiogenesis (22). On the basis of these considerations, we propose a common mechanism of action *in vivo* for the known antiangiogenic protein fragments: they polymerize an arginine-glycine-aspartic acid-containing protein, the resulting polymers bind to the $\alpha v \beta 3$ integrin on angiogenic endothelial cells, and the polymers inhibit cell proliferation and cause apoptosis. The polymers possibly act by affecting ECM formation (23) or by arginine-glycine-aspartic acid-mediated activation of intracellular caspases (24, 25).

We thank Dr. Eva Engvall for comments on the manuscript. This work was supported by Grant CA74238 and Cancer Center Support Grant CA30199 from the National Cancer Institute, and Grant DAMD17-00-1-0556 from the Department of Defense.

- Hynes, R. O. (1990) in *Fibronectins* (Springer, New York).
- Clark, E. A. & Brugge, J. S. (1995) *Science* **268**, 233–239.
- Giancotti, F. G. & Ruoslahti, E. (1999) *Science* **285**, 1028–1032.
- Frisch, S. M. & Ruoslahti, E. (1997) *Curr. Opin. Cell Biol.* **9**, 701–706.
- Morla, A., Zhang, Z. & Ruoslahti, E. (1994) *Nature (London)* **367**, 193–196.
- Chernousov, M. A., Fogarty, F. J., Kotliansky, V. E. & Mosher, D. F. (1991) *J. Biol. Chem.* **266**, 10851–10858.
- Morla, A. & Ruoslahti, E. (1992) *J. Cell Biol.* **118**, 421–429.
- Sechler, J. L., Takada, Y. & Schwarzbauer, J. E. (1996) *J. Cell Biol.* **134**, 573–583.
- Hocking, D. C., Smith, R. K. & McKeown-Longo, P. (1996) *J. Cell Biol.* **133**, 431–444.
- Pasqualini, R., Bourdoulous, S., Woods, V., Koivunen, E. & Ruoslahti, E. (1996) *Nat. Med.* **2**, 1197–1203.
- Arap, W., Pasqualini, R. & Ruoslahti, E. (1998) *Science* **279**, 377–380.
- Hanahan, D. & Folkman, J. (1996) *Cell* **86**, 353–364.
- O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. Q., Mose, M., Lane, W. S., Cao, Y., Sage, E. H. & Folkman, J. (1994) *Cell* **79**, 315–328.
- O'Reilly, M. S., Boehm, T., Shing, Y., Fukui, N., Casas, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R. & Folkman, J. (1997) *Cell* **88**, 277–285.
- Homandberg, G. A., Williams, J. E., Grant, D., Schumacher, B. & Eisenstein, R. (1985) *Am. J. Pathol.* **120**, 327–332.
- Homandberg, G. A., Kramer-Bjerke, J., Grant, D., Christianson, G. & Eisenstein, R. (1986) *Biochim. Biophys. Acta* **874**, 61–71.
- O'Reilly, M. S., Pirie-Shepherd, S., Lane, W. S. & Folkman, J. (1999) *Science* **285**, 1926–1928.
- Kost, C., Benner, K., Stockmann, A., Linder, D. & Preissner, K. (1996) *Eur. J. Biochem.* **236**, 682–688.
- Miosge, N., Sasaki, T. & Timpl, R. (1999) *FASEB J.* **13**, 1743–1750.
- Ill, C. R. & Ruoslahti, E. (1985) *J. Biol. Chem.* **260**, 15610–15615.
- Ruoslahti, E. (1996) *Annu. Rev. Cell Dev. Biol.* **12**, 697–715.
- Brooks, P. C., Clark, R. A. & Cheresh, D. A. (1994) *Science* **264**, 569–571.
- Bourdoulous, S., Orend, G., MacKenna, D. A., Pasqualini, R. & Ruoslahti, E. (1998) *J. Cell Biol.* **143**, 267–276.
- Buckley, C. D., Pilling, D., Henriquez, N. V., Parsonage, G., Threlfall, K., Scheel-Toellner, D., Simmons, D. L., Akbar, A. N., Lord, J. M. & Salmon, M. (1999) *Nature (London)* **397**, 534–539.
- Adderley, S. R. & Fitzgerald, D. J. (2000) *J. Biol. Chem.* **275**, 5760–5766.